

Whole Blood Thrombin: Development of a Process for Intra-Operative Production of Human Thrombin

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Abstract: Thrombin-based clotting agents currently used for topical hemostasis with absorbable sponges, fibrin sealants, and platelet gels are primarily derived from bovine or pooled human plasma sources. Autologous thrombin has important safety advantages in that it does not carry the same safety concerns as pooled plasma-derived products and it avoids exposure to risks associated with bovine-derived proteins. The goal of our research was to develop a rapid, reliable, and simple to perform process to generate autologous human thrombin in the intra-operative setting, from patient whole blood as the starting source material. Using whole blood instead of plasma as the starting material, it is possible to avoid the inherent delay in thrombin availability associated with a primary step of plasma isolation. In this study, we varied several key processing parameters to maximize thrombin production, reproducibility and stability. Autologous thrombin production was generated using a dedicated, single use disposable with a sterile reagent. The disposable consists of a tubular reaction chamber containing glass microsphere beads to activate the alternative pathway of the coagulation cas-

cade. At the end of the process, thrombin-activated serum was harvested from the reaction chamber. The average activity of the thrombin produced at room temperature by this system was 82.8 ± 15.9 IU/mL. The total processing time was <30 minutes. The system was compatible with Anticoagulant Citrate Dextrose-Solution A (ACD-A) (8%-12%). The average volume of thrombin harvested from each aliquot of blood was 7.0 ± 0.3 mL, and the stability of thrombin was observed to be temperature dependent, with cold storage better preserving thrombin activity. Clot times with platelet concentrates at 1:4.3 and 1:11 ratios (thrombin to platelet concentrate) were <10 and 20 seconds, respectively. A process for the preparation of thrombin from whole blood, under conditions compatible with the resources of an operating room, has been developed. The device is simple to use, requires 30 minutes, and can consistently produce thrombin solutions that achieve rapid clotting of platelet concentrates, plasma, and fibrinogen concentrates even when mixed at thrombin to blood product ratios of 1:11. **Keywords:** blood, human thrombin, stability, fibrinogen, clot. JECT. 2007;39:18-23

Thrombin (activated factor II) is a coagulation protein that has many effects in the coagulation cascade. It is a serine protease that converts soluble fibrinogen to an active form that assembles into fibrin (1). Thrombin also activates factor V, factor VIII, factor XI, and factor XIII. This positive feedback accelerates the production of thrombin. Factor XIIIa is a transglutaminase that catalyzes the formation of covalent bonds between lysine and glutamine residues in fibrin. The covalent bonds increase the stability of the fibrin clot.

Thrombin has been widely used in various surgical procedures for the reduction of bleeding in combination with collagen sponges (2-5). A combination of thrombin with a

concentrated fibrinogen source (i.e., single donor cryoprecipitate or purified fibrinogen from pooled plasma) is also commonly used in clinical practices as an adjunct to achieve hemostasis (6). In addition to its activity in the coagulation cascade, thrombin also promotes platelet activation through activation of protease-activated receptors on the platelet. This activation pathway and clot formation has led to the use of thrombin in combination with platelet concentrates to form platelet gels that are being used in orthopedic, oral, and maxillofacial surgery as a means to enhance wound healing. It is proposed that bone growth can be enhanced because of increased level of tissue growth factors associated with platelet granules (7-10).

The choice for the source material to derive thrombin used in surgical procedures is extremely important for the resulting thrombin risk profile to the patient. The safest thrombin source is a patient's own blood. However, the majority of thrombin used today is of bovine origin. The immunologic response elicited by bovine thrombin prepa-

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rations and their clinical sequela are well established. Because bovine thrombin preparations are not completely pure, an immunologic response can be elicited by other bovine plasma contaminants such as factor V. The bovine-elicited antibodies have the potential to cross-react with their corresponding human factors. For example, some of the most serious adverse effects are mediated by antibodies with cross-reactivity to human factor V. These factor V reactive antibodies can cause a range of coagulopathy-associated symptoms that may lead to adverse reactions, including severe and life-threatening bleeding (11,12). Another concern particularly for thrombin sourcing from bovine and pooled human products is possible exposure to prions causing variant Creutzfeldt-Jacob disease (vCJD) in humans (13). Thus, the most preferred source material for preparing thrombin used in surgical procedures is autologous thrombin.

Until recently, most of the commercially available thrombin preparations were derived from large pools of mixed donor plasma by Cohn-Oncley fractionation (14). Active thrombin was produced from purified prothrombin using several different methods including use of different coagulation proteins (15,16) or snake venom as activators (17-20). Although these methods are well adapted for industrial scale production of thrombin from large pools of plasma, they are not practical methods for the preparation of single donor autologous thrombin.

We have previously reported the development of a simple and reliable method for development of autologous thrombin from single donor plasma (21). Thrombin production can be readily initiated by adding an excess of calcium ions to citrated plasma, which allows initiation of the clotting cascade and finally to the production of thrombin. However, the use of thrombin prepared in this simple manner is of limited value because of the slow rate of thrombin production and the low concentration of thrombin produced. Our method overcomes these limitations by using glass beads to provide a negatively charged surface to greatly accelerate the rate of thrombin production. Furthermore, the use of the thrombin reagent allows the stabilization of the thrombin by blocking the inhibitory actions of protein C, protein S, and particularly, anti-thrombin III (1). The resulting thrombin is sufficiently stable that it can be stored for hours after its production and still maintain clinical use.

In this study, we have developed a reliable technique to generate autologous human thrombin in the intra-operative setting from whole blood instead of plasma as the starting source material within a 30-minute period. With this method, it is possible to avoid a delay in thrombin availability to the surgical team caused by elimination of a plasma separation step and requires only 12 mL of whole blood. The goal of this research was to optimize the thrombin production process using whole blood and to

characterize the thrombin produced for activity and stability. The performance characteristics of the thrombin were evaluated both quantitatively by measuring thrombin activity using a functional clot assay and by measuring the ability and time required for the thrombin to complete gel formation with platelet-rich plasma (PRP).

MATERIALS AND METHODS

Collection of Anticoagulant Citrate Dextrose-Solution A Blood

Fresh blood was obtained from our in-house donation program. The blood was collected in 60-mL Anticoagulant Citrate Dextrose-Solution A (ACD-A) (8%) anticoagulated syringes obtained from pre-screened healthy volunteers. The blood was handled according to established procedures for the collection and processing of human blood products. Each blood unit was processed within 6 hours of initiation of the collection.

Blood Thrombin Processing System

The blood thrombin processing system (BTPS) consists of two parts: a tubular reaction chamber containing negative surface charge beads needed for initiation of thrombin formation and a reagent consisting of calcium chloride and ethanol at concentrations of 25 mmol/L and 66%, respectively.

Preparation of Thrombin

Twelve milliliters of blood and 4 mL thrombin reagent were added to the reaction chamber. The contents were mixed and incubated for 15 minutes. At the end of the 15-minute incubation period, the blood/beads were mixed by agitation to break any formed clots, followed by a centrifugation step for 12 minutes at 2100g. At the end of the centrifugation step, thrombin activated serum (top fluid) was harvested from the reaction chamber.

To establish intra-variability of the thrombin production system, the same blood was used with 10 different thrombin-producing systems. Inter-variability was assessed by determining the thrombin activity in 20 thrombin-producing systems from different blood units. To study the influence of ambient temperature on thrombin production, the process was performed at two different temperatures: 17°C and 25°C.

Preparation of PRP

PRP was prepared using 60-mL ACD-A anticoagulated blood using the Magellan device as per the manufacturer's instructions.

Thrombin Activity and Quantitative Assessment of Thrombin

Thrombin activity was analyzed using the modified Clauss method (22) as described previously (21). Briefly,

200 μL of a 2.0-mg/mL pre-warmed (37°C) solution of fibrinogen (catalog F-4883; Sigma, St. Louis, MO) was added to 100 μL of the thrombin sample. The time required for clot formation was recorded using a fibrometer (Fibro System; Becton-Dickinson, Franklin Lakes, NJ). The thrombin activity in the samples was determined by correlating the time to clot formation to a standard curve generated with titrations of thrombin (catalog 50502; Biopool US, Ventura, CA). Because each time-point on the standard curve corresponds to the thrombin activity needed to clot the standard concentration of fibrinogen, the thrombin activity in an unknown sample can be extrapolated from the time to clot formation.

Clot Time and Qualitative Assessment of Thrombin

Clot times were assessed by mixing PRP with variable concentrations of thrombin. Thrombin preparations of different activity used for this test were prepared by serial dilutions of the starting thrombin with saline solution. One part of thrombin and 3.3 or 10 parts of PRP were mixed in a cup, and the time to clot formation was assessed. To establish the relationship between clot time and thrombin activity, both analyses were performed, and a correlation was established.

Assessment of Stability

The stability of the produced thrombin was assessed by storing 3 mL of thrombin either at the production temperature (i.e., 17°C or 25°C) or at 4°C . Thrombin activity was assessed at time 0 (after preparation) and after 2 and 4 hours of storage.

Statistical Analysis

All results are presented as mean and SD. Coefficient of variation (CV) was calculated for intra- and inter-variability. Significance between groups was assessed using the Student *t* test.

RESULTS

Intra-Reproducibility of Thrombin Produced at 25°C

To establish the intra-variability of the thrombin production system, the same blood was used with 10 different thrombin-producing systems. Using blood from the same unit and 10 different BTPSs (intra-reproducibility), the thrombin activity was 82.8 ± 15.9 IU/mL, with a CV of 21% (Table 1).

Inter-Reproducibility of Thrombin Produced at 25°C

Inter-variability was assessed by determining the thrombin activity in 20 thrombin-producing systems from different blood units. The inter-reproducibility was established from 10 different blood units, using BTPSs from the same lot of material. Thrombin activity was found to be $67.9 \pm$

Table 1. Thrombin production at 24°C : intra-reproducibility of thrombin produced from whole blood using the BTP system.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean \pm SD ($n = 10$)	7.0 ± 0.3	82.8 ± 15.2
CV	4%	21%
Range (min – max)	6–7	59–104

Thrombin activity is expressed as mean \pm SD.

19 IU/mL (range, 47–117 IU/mL), with a CV of 32% (Table 2).

Short-Term Stability of Thrombin Produced at 25°C

The results are shown in Table 3. For thrombin prepared at 25°C , the activity was 67.9 ± 19.0 IU/mL, with a CV of 32%. When samples were stored at 4°C for 4 hours, the thrombin activity was 59.8 ± 15.9 IU/mL, with a CV of 31%. The data also showed that thrombin prepared at normal ambient temperature (25°C) is sufficiently stable to effectively form fibrin clots and platelet gels when stored up to 4 hours at 4°C .

Correlation Between Thrombin Activity and Clot Time With PRP

Thrombin activity and clot times were analyzed in 83 samples. Clot formation was analyzed by mixing 1 part of thrombin and 3.3 or 10 parts of PRP. Results for thrombin activity and in vitro clot times with PRP (1 part of thrombin + 3.3 parts of PRP) using different blood units are shown in Figure 1. Data from this study show that acceptable clot times (≤ 15 seconds) with PRP can be achieved by mixing 1 part of thrombin + 3.3 parts of PRP. Results suggest that a minimum thrombin activity of 15–20 IU/mL will be needed to achieve acceptable clot times. There was no significant difference in clot times between units with a thrombin activity of 30–45 IU/mL compared with units with a thrombin activity of 46–80 IU/mL (8.2 ± 1.2 and 7.7 ± 1.5 seconds, respectively; no significant difference). Units with a thrombin activity of 0–30 IU/mL had significantly longer clot times compared with units with a thrombin activity of 30–45 IU/mL (15.0 ± 6.4 and 8.2 ± 1.4 seconds, respectively; $p < .0001$). The total population ($n = 64$) with a thrombin activity of >30 IU/mL had 7.9 ± 1.5

Table 2. Thrombin production at 24°C : inter-reproducibility of thrombin produced from whole blood using the BTP system.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean \pm SD ($n = 20$)	7 ± 0.4	67.9 ± 19.0
CV	7%	28%
Range (min – max)	6–7	47–117

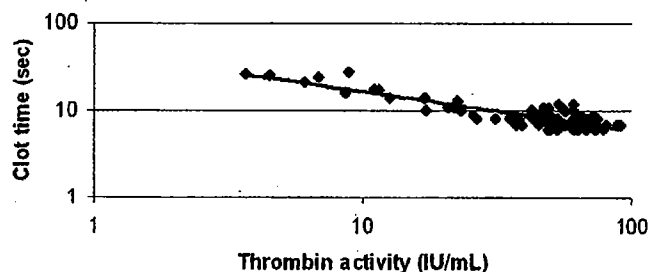
Thrombin activity is expressed as mean \pm SD.

Table 3. Short-term stability of thrombin produced at 24°C from whole blood stored at 4°C.

	Thrombin Activity (IU/mL)	
	T ₀	T ₄ on Ice
Mean ± SD (n = 20)	67.9 ± 19.0	59.8 ± 15.9
CV	32%	31%
Range (min - max)	47-117	45-102

Thrombin activity is expressed as mean ± SD.

Correlation Between Thrombin activity and in-vitro clot time with PRP (1:4.3 ratio)

**Figure 1.** Correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 3.3 parts of PRP) using different blood units.

seconds in clot time. Data from this experiment suggest that clot times with platelets concentrates at 1:4.3 ratios were <10 seconds.

Results for correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 10 parts of PRP) using different blood units are shown in Figure 2. Units with a thrombin activity of 0-30 IU/mL had significantly longer clot times compared with units with a thrombin activity of 30-45 IU/mL (23.1 ± 4.8 and 14.2 ± 2.6 , respectively; $p < .0001$). The total population ($n = 45$) with a thrombin activity of >30 IU/mL had 12.3 ± 1.8 seconds in clot time. Results suggest that a minimum thrombin activity of 30 IU/mL will be needed to achieve acceptable clot times (≤ 15 seconds).

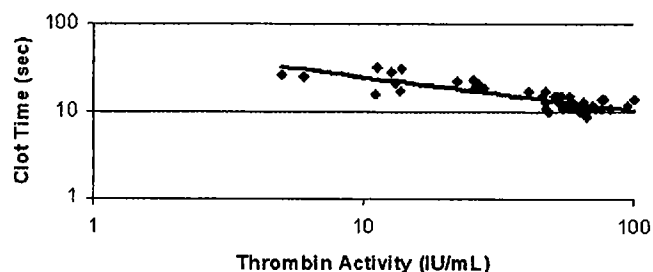
Quality of BTPS-Thrombin Produced at Different Temperatures

The ambient temperature in the operating room, where the whole blood thrombin would be produced, can vary; hence, we assessed the quality of thrombin at 17°C and 25°C (corresponding to 63°F and 77°F). Within 10 minutes of production, there was significant difference in the thrombin activity between thrombin produced at 17°C and 25°C (66 ± 20 and 77 ± 30 IU/mL, respectively; $p > .05$).

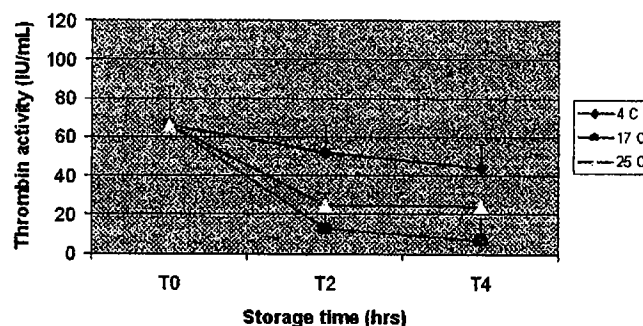
Stability of BTPS-Produced Thrombin

The stability of the BTPS-produced thrombin was assessed by measuring thrombin activity at 2 and 4 hours

Correlation between thrombin activity and in-vitro clot time with PRP (1:11)

**Figure 2.** Correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 10 parts of PRP) using different blood units.

Thrombin production at 17°C & storage at 4, 17 & 25°C

**Figure 3.** Stability of thrombin produced using the BTPS. The thrombin was produced at 17°C and stored for 2 and 4 hours at different temperatures (4°C, 17°C, and 25°C). Thrombin activity was analyzed by the method as described in the Materials and Methods.

after production. The stability was determined at the production temperature (17°C and 25°C, respectively) and at 4°C.

Stability of BTPS-Thrombin Produced at 17°C

Thrombin produced at a lower ambient temperature (17°C) did not retain its thrombin activity as well as thrombin produced at higher ambient temperatures. After 4 hours of storage at 4°C, the thrombin activity had decreased to 44 ± 11.0 IU/mL. After 4 hours of storage at 17°C and 25°C, we found that the thrombin activity decreased to 8 ± 0.1 and 24.2 ± 12.7 IU/mL, respectively (Figure 3).

Stability of BTPS-Thrombin Produced at 25°C

When thrombin was prepared at normal ambient temperature (25°C) and stored at that temperature, the thrombin activity declined to 47.2 ± 25.2 IU/mL after 4 hours (Figure 4). However, when the same thrombin was stored at 4°C, the decrease in thrombin activity was not as pronounced as when stored at 17°C (74.8 ± 26.9 and 36.8 ± 21.2 IU/mL, respectively) after 4 hours. The data suggest

Thrombin production at 25°C & storage at 4, 17 & 25°C

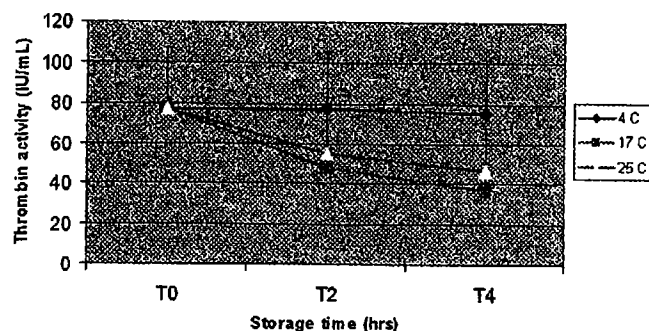


Figure 4. Stability of thrombin produced using the BTPS. The thrombin was produced at 25°C and stored for 2 and 4 hours at different temperatures (4°C, 17°C, and 25°C). Thrombin activity was analyzed by the method as described in the Materials and Methods.

that thrombin prepared at normal ambient temperature (25°C) can be stored for up to 4 hours at 4°C and still maintain its effectiveness.

DISCUSSION

The results of this study indicate that a practical process for the routine production of human thrombin from autologous whole blood in the intra-operative setting can be achieved. The whole blood technique we developed is derived from the method that we previously commercialized using single donor plasma. Thrombin used in surgical procedures as an adjunct to hemostasis (3–10) is primarily derived from bovine sources and is associated with major concerns about adverse reactions, e.g., antibody formation against human FV leading to bleeding episodes (11,12) and transmission of bovine prions possibly causing vCJD (13). Use of autologous thrombin is an attractive alternative choice in surgeries because it avoids these infectious disease and immunogenicity risks. This whole blood procedure provides a faster method than our previous plasma procedure because it eliminates the need for a centrifugation and harvest step for first collecting the plasma. The entire procedure can readily be performed in ~30 minutes. In this study, we evaluated the stability of thrombin produced from human whole blood. The blood we used was fresh blood collected from normal donors and tested within 8 hours of collection to replicate intended use conditions. In surgery, the blood will be taken directly from the patient, and thrombin will be produced intra-operatively.

The enzymatic activity of thrombin to be used either by itself or in combination with fibrinogen or platelets is important for the speed of clot formation (23). Typically for most applications, bovine thrombin is diluted to 500 and 1000 IU/mL. The thrombin produced in this study ranged in activity from 59 to 104 IU/mL, with a mean of 83 IU/

mL. This thrombin activity is very similar to our previous observation with plasma using a thrombin processing device (TPD) (21). In addition, we have previously shown that TPD-produced thrombin can activate platelets to release α -granule content, and consequently, growth factors, comparably with commercially available thrombin (24).

When 10 units of BTPS-thrombin were produced from the same unit of blood, the CV was 21%, indicating the ability of the BTPS to produce thrombin with consistent thrombin activity. Furthermore, when blood from different donors was used, the CV increased to 28%. Thus, there is variability between donors/blood in the ability to produce active thrombin using the BTPS. This would be expected because availability of prothrombin and other coagulation factors needed for formation of the prothrombinase complex can vary between donor blood. The results suggest that the BTPS is able to produce relatively consistent thrombin with acceptable activity.

Using *in vitro* clot time with PRP as a qualitative measure of the efficacy of the thrombin, we found that the clot time depends on the ratio of thrombin to PRP. In most cases, a clot time of 10–15 seconds was equal to instant clotting, because of the manual variability of the test. Data from this study showed that clot times with platelet concentrates at 1:4.3 and 1:11 ratios were <10 and 20 seconds, respectively. This *in vitro* clot time suggests that lower levels of thrombin activity than what is commonly used today would be sufficient for adequate clot formation *in vivo*. In fact, using a combination of thrombin and fibrinogen in a fibrin sealant, it has been shown that a thrombin with an activity of 50–100 IU/mL had a higher adhesiveness compared to one with a thrombin activity of 500 IU/mL (25).

Typically enzymes are sensitive to temperature, and the rate of activation and degradation is dependent on the specific optimal temperature for each enzyme. Our data suggested that thrombin produced at 24°C had a higher activity and retained its activity for a longer time compared with thrombin produced at a lower temperature (17°C). This observation is very important for the user. Because thrombin is an active enzyme, the user has to be aware of both the production temperature (the ambient temperature in the room) and the storage temperature for optimal efficacy of the product. For example, if the ambient temperature is 25°C, the BTPS-thrombin can be stored for up to 2 hours without any special precautions, i.e., it can be stored on the bench. If longer storage time is needed, the thrombin should be stored at 4°C. However, if the ambient temperature is in the 16–18°C range, as is the case in certain surgical procedures, the shelf-life of the thrombin is shorter, and it should be used within 1–2 hours of production or stored on ice.

In conclusion, we have demonstrated that it is practical to produce active and effective thrombin from whole

blood at low and normal ambient temperatures. We have also showed that the activity and in vitro clot times of thrombin are dependent on both the production and storage temperatures. Thrombin produced at 17°C and 25°C retained clotting efficacy for 4 hours when stored at 4°C. Thus, we have developed a simple, rapid, and reliable method for generation of stable thrombin from the patients' own blood in ~30 minutes.

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